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13. Monkey COS7 cells were transfected with purified plasmid DNA (2.5 µg/ml) by the DEAE dextran method (2). The cells were stained with B-rIL-6 after culture for 2 days. For the preparation of B-rIL-6, rIL-6 (5 µg) (12) in 10 µl of 0.1M bicarbonate buffer (pH 8.4) was incubated with 1.3 µg of biotin-O-succinimide ester (Bioscience #B-1000) in dimethyl sulfoxide (DMSO) (2 mg/ml) at room temperature for 3 hours, and free biotin was separated by Sephadex G-50 column. COS7 cells (5×10^5) transfected with DNA were incubated with ~10 ng of B-rIL-6 in 10 µl of biotin-free RPMI 1640 (Deficient RPMI 1640, Irvine Scientific) at 4°C for 90 min. The cells were washed by passing through fetal calf serum and further incubated with FITC-A at 4°C for 20 min. Positively stained cells were sorted out with a FACS 440 (Becton Dickinson) as described (23).
- Escherichia coli MC1061/P3 was transformed with episomal DNA collected from the sorted cells as described (12). Plasmid DNA was extracted and subjected to a second round of sorting as described above. Single colonies (250) were randomly picked after four rounds of selection, and plasmid DNA was extracted from each colony. Plasmid DNA with insert cDNAs greater than 2 kb was individually transfected into COS7 cells and the expression of the IL-6-R was analyzed by flow cytometry. COS7 cells transfected with plasmid DNA from two colonies were found to express the IL-6-R. One of them, pBSF2R.236, was extensively analyzed.
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A Monoclonal Antibody to the α Subunit of G_k Blocks Muscarinic Activation of Atrial K^+ Channels

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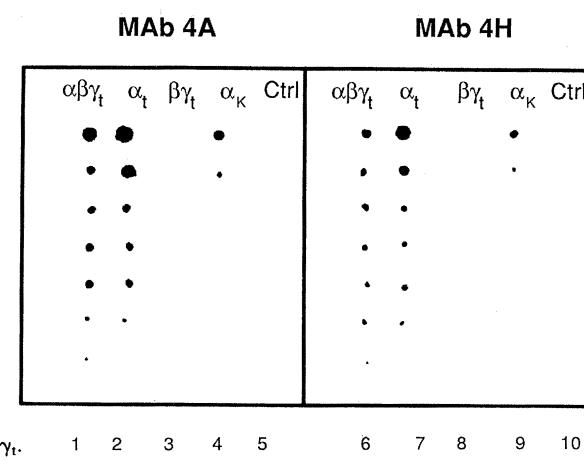
The activated heterotrimeric guanine nucleotide binding (G) protein G_k , at subpicomolar concentrations, mimics muscarinic stimulation of a specific atrial potassium current. Reconstitution studies have implicated the α and $\beta\gamma$ subunits as mediators, but subunit coupling by the endogenous G protein has not been analyzed. To study this process, a monoclonal antibody (4A) that binds to α_k but not to $\beta\gamma$ was applied to the solution bathing an inside-out patch of atrial membrane; the antibody blocked carbachol-activated currents irreversibly. The state of the endogenous G_k determined its susceptibility to block by the antibody. When agonist was absent or when activation by muscarinic stimulation was interrupted by withdrawal of guanosine triphosphate (GTP) in the presence or absence of guanosine diphosphate (GDP), the effects of the antibody did not persist. Thus, monoclonal antibody 4A blocked muscarinic activation of potassium channels by binding to the activated G protein in its holomeric form or by binding to the dissociated α subunit.

COUPLING BETWEEN THE ATRIAL muscarinic acetylcholine receptor and the potassium channel current (K_{ACh}) that it activates (1) is thought to be independent of a cytoplasmic second messenger pathway (2). G proteins were implicated as coupling agents because whole-cell K_{ACh} currents required guanosine triphosphate (GTP), were decreased by pertussis toxin (PTX) (3), and became independent of ligand in the presence of the nonhydrolyzable GTP analog 5'-guanylylimidodiphosphate (GppNHp) (4). The muscarinic effects on the atrial membrane potential and atrial pacing were also blocked by PTX (5). Taken together with the results in (2), the

possibility of G protein mediation independent of cyclic nucleotide second messengers was noted (3). Analysis of the whole-cell currents may be complicated, however, by the presence of a PTX-insensitive nonselective cation current related to phosphoinositide hydrolysis (6). The demonstration that another nonhydrolyzable GTP analog, gua-

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Fig. 1. "Dot blots" of the reaction between mAbs 4A and 4H (13) and transducin holoprotein $G_t(\alpha\beta\gamma_t)$ (lanes 1 and 6), α_t -GTP γ S (lanes 2 and 7), the $\beta\gamma$ subunit of transducin ($\beta\gamma_t$) (lanes 3 and 8), α_k -GTP γ S (lanes 4 and 9), and control protein (trypsin inhibitor) (lanes 5 and 10). Dot blots were performed as described in (12), and antibody binding was detected by 125 I-labeled protein A binding and autoradiography of dot blots. The mAb 4A has a similar affinity for $\alpha\beta\gamma_t$ and α_t , lower affinity for α_k , and no affinity for $\beta\gamma_t$.



nosine 5'-(γ -thio)triphosphate (GTP γ S), activated single channel K_{ACh}^+ currents in excised inside-out membrane patches was the best indication of direct gating (7), but effects from membrane-associated mediators such as protein kinase C could not be excluded, nor was there direct evidence that a G protein could mediate the response. Then, picomolar concentrations of a specific exogenous G protein, preactivated with GTP γ S, were shown to produce activation, and the activation was unaffected by adenosine triphosphate (ATP) or by phorbol ester in the presence of ATP (8). The G protein was called G_k , a term previously used for the endogenous G protein (4), and this membrane-delimited effect was assumed to be direct (8, 9).

The phenomenon of direct G protein gating may be widespread. Direct gating occurs in the coupling of another type of K^+ channel to muscarinic acetylcholine and somatostatin receptors in clonal anterior pituitary (GH_3) cells (10), and G_s , the stimulatory regulator of adenylyl cyclase, directly gates two distinct dihydropyridine-sensitive Ca^{2+} channels that are coupled to β -adrenergic receptors in cardiac and skeletal muscle, respectively (11). Preactivated α subunits are equipotent to the holomeric G proteins and have been proposed to mediate the effects (8–10). To examine the coupling process in heart under more physiological circumstances, we used monoclonal antibodies (mAbs) raised against the α subunit α_t of frog transducin (12, 13). Two of these, mAb 4A and mAb 4H, cross-react with the α subunit of G_k , α_k (Fig. 1), and one of them (mAb 4A) inhibits the interaction of α_t with rhodopsin, thereby blocking transducin activation by light (12).

We activated single atrial K_{ACh}^+ channel currents i_k , with the muscarinic agonist carbachol at 0.1 μM in the patch pipette. When the membrane patch is excised in the inside-out configuration (IO), activation persists if GTP is present in the bath solution. Inclusion of mAb 4A in the bath blocked carbachol activation of i_k ; the block was not due to a general property of immunoglobulins because control immunoglobulin G (IgG) had no effect (Fig. 2A). The mAb 4H binds α_t on a site distinct from mAb 4A but has no effect on its activation by light (12) so we tested the effects of 4H as well. Like IgG, it was ineffective on i_k . In the presence of IgG or 4H, the single channel currents were steady for at least 10 min and for as long as about 30 min. The mAb 4A at 500 nM reduced i_k within 10 s of application; the effects became steady after about 2 min. The remaining single channel currents (Fig. 3B) appeared unchanged, and this was confirmed by the histograms of amplitude and

open times, which in the presence of mAb 4A were identical to control (Fig. 3C). The effect of the antibody was therefore entirely on opening frequency.

The block of muscarinic activation by mAb 4A was not relieved by increasing the GTP concentration from 100 μM to 1 mM or by washing with mAb 4A-free bathing solution for up to 20 min. Subunit α_k activated with GTP γ S (α_k^*) (10 pM) added to the bath after washing out mAb 4A did reactivate an i_k that had the same frequency distributions of open times, burst durations, and amplitudes as carbachol-activated i_k (14). Reactivation with α_k^* was not possible in the presence of excess mAb 4A ($n = 4$) (Fig. 2A). Reactivation consisted of restora-

tion of single channel currents that were unchanged in amplitude or dwell times from those present prior to block (Fig. 2C). When mAb 4A was added after α_k^* , it was no longer effective in blocking i_k . We stimulated i_k with α_k^* and then added mAb 4A on four other occasions with the same result; once i_k was stimulated by α_k^* , mAb 4A block was no longer possible. This was independent of the continued presence of α_k^* in the bath, that is, washing α_k^* from the bath did not affect the outcome. This is consistent with our observations that α_k^* acts irreversibly on i_k (8–10, 14).

To test whether mAb 4A block was possible after irreversible activation of the atrial membrane G protein, we added GTP γ S at

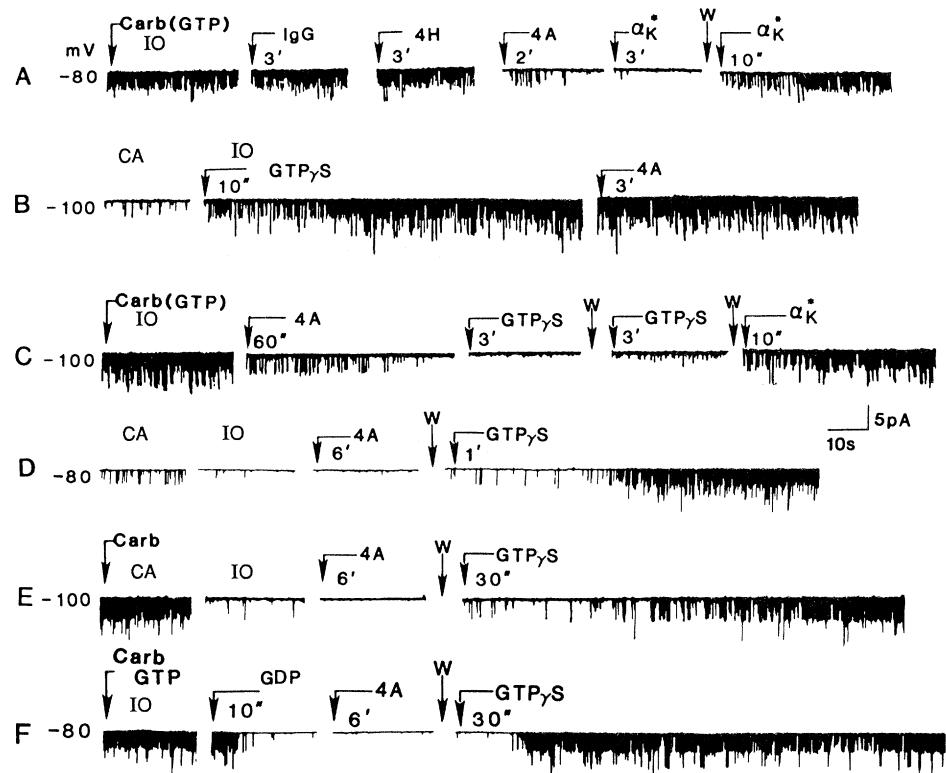


Fig. 2. Characteristics of mAb 4A block of single K^+ channel currents, i_k , in inside-out (IO) patches excised from atria of adult guinea pigs. Carbachol (Carb) (0.1 μM) in the patch pipette and GTP (100 μM) in the bathing solution activated i_k . The single channel currents were recorded with symmetrical isotonic K^+ (140 mM) solutions (25), and the holding potentials are indicated. (A) Slow time resolution recordings of test agent effects. Times on top of each trace are minutes ('') or seconds (") elapsed between the addition of the test agent to the bathing solution and the illustrated recording. mAb 4H (6.5 μM) and control IgG (Sigma) (6.5 μM) had no effects, but mAb 4A (6.5 μM) blocked i_k . α_k^* (10 pM) (12) added in the presence of mAb 4A was also ineffective, but after washing out mAb 4A (W), α_k^* activated i_k . (B) mAb 4A (6.5 μM) did not block i_k previously activated by GTP γ S at 100 μM . The membrane patch shows little baseline activity in the cell-attached (CA) configuration in the absence of carbachol. After IO excision GTP γ S (100 μM) stimulated i_k . mAb 4A was ineffective in blocking this effect. (C) mAb 4A applied prior to GTP γ S prevents activation. i_k in the IO patch were stimulated by carbachol (0.1 μM) in the patch pipette and GTP (100 μM) in the bath. Prior incubation with mAb 4A (6.5 μM) prevented the GTP γ S response shown in (B). After washing out mAb 4A, GTP γ S (100 μM) was ineffective, but α_k^* reactivated i_k . Hence, mAb 4A blocked endogenous atrial G_k irreversibly. (D) mAb 4A does not block endogenous atrial G_k that is not activated. Spontaneous i_k was reduced in mAb 4A after excision into bathing solution containing 100 μM GTP. Unlike in (C), however, GTP γ S (100 μM) activated i_k currents after washing away mAb 4A. (E) mAb 4A does not block endogenous atrial G_k when the activated muscarinic acetylcholine receptor and atrial G_k are tightly coupled (15). After activation by carbachol (0.1 μM), the patch is excised into GTP-free solution, and i_k disappears. As in (D), but again unlike in (C), GTP γ S activated i_k after washing away mAb 4A. (F) mAb 4A does not block endogenous atrial G_k when the muscarinic receptor and atrial G_k are dissociated in the presence of GDP (16). As in (D) and (E) GTP γ S activated i_k after washing away mAb 4A.

100 μM to the bath after excising an atrial patch, this time without carbachol in the patch pipette. The mAb 4A was ineffective (Fig. 2B), and this result was repeated in three other experiments. However, prior block of carbachol-activated i_K with mAb 4A prevented any stimulation of i_K by subsequent activation of endogenous atrial G

protein with GTP γ S at 100 μM (Fig. 2C). Unlike α_k^* , which was always effective after washing mAb 4A from the bath, GTP γ S was ineffective in three of five instances and had a slight transient effect in two of five cases.

Although mAb 4A blocked muscarinic stimulation of i_K (Fig. 2C), it did not pro-

duce a persistent block when membrane G_K was quiescent either because agonist was absent or because agonist was ineffective due to absence of GTP. In four experiments mAb 4A was added under conditions where endogenous G_K molecules were not stimulated by agonist and were traversing the GTP-GDP cycle at a slow basal rate, with most of the G_K in the inactive "GDP" form (Fig. 2D). In three other experiments, mAb 4A was added under conditions (GTP absent, Mg^{2+} added) (Fig. 2E) in which receptors were in a high-affinity state for agonist with the G protein tightly coupled to the muscarinic receptor (15). In an additional three experiments GDP at 100 μM was present in the bath and the receptor in its low-affinity state was dissociated from the unactivated G protein (16) (Fig. 2F). In all situations GTP γ S, formerly ineffective after mAb 4A block of muscarinic stimulation, could now activate i_K (compare Fig. 2C with Figs. 2, D through F).

Monoclonal antibody 4A blocked carbachol-activated i_K in a concentration-dependent manner with effects appearing at concentrations as low as 650 pM (Fig. 3) and becoming steady between 0.5 and 3 min after application. Because of the stochastic nature of single channel currents, the effects were quantified by time averaging the openings (1). Data were integrated every 200 to 500 ms to produce the diaries of proportion of open time (P) for the N channels in the patch (Fig. 3B) ($n = 2-5$). The diaries were averaged for 20 to 30 s (Fig. 3D), and the average value at each concentration of mAb 4A was normalized and plotted as a concentration-response curve (Fig. 3E). The curve was fit as an adsorption isotherm and had a dissociation constant (K_d) of 65 nM.

Our results show that guinea pig atrial G_K , activated by muscarinic agonist and GTP, and human erythrocyte α_K , activated by GTP γ S, share a common antigenic determinant recognized by mAb 4A. Monoclonal antibody 4A was raised against α_t and does not interact with $\beta\gamma_t$ (Fig. 1), which is similar to the $\beta\gamma$ subunits of other G proteins (17), yet mAb 4A blocks exogenous α_k^* . Hence mAb 4A probably interacts with the α subunit of atrial G_K and not its $\beta\gamma$ subunits. Although mAb 4A reacts more strongly with α_k^* than with α_K , α_k^* has no effect on atrial K_{ACh}^+ channels, even at concentrations 10,000 times as great as threshold concentrations of α_k^* ($n = 3$). Thus, the antigenic site itself, if it interacts with the channel, is not sufficient to trigger a response. Our results also show that the antigenic site is not always exposed to mAb 4A or, if it is, bound mAb 4A sometimes cannot produce functional effects. First, mAb 4A had no effect after GTP γ S activated the

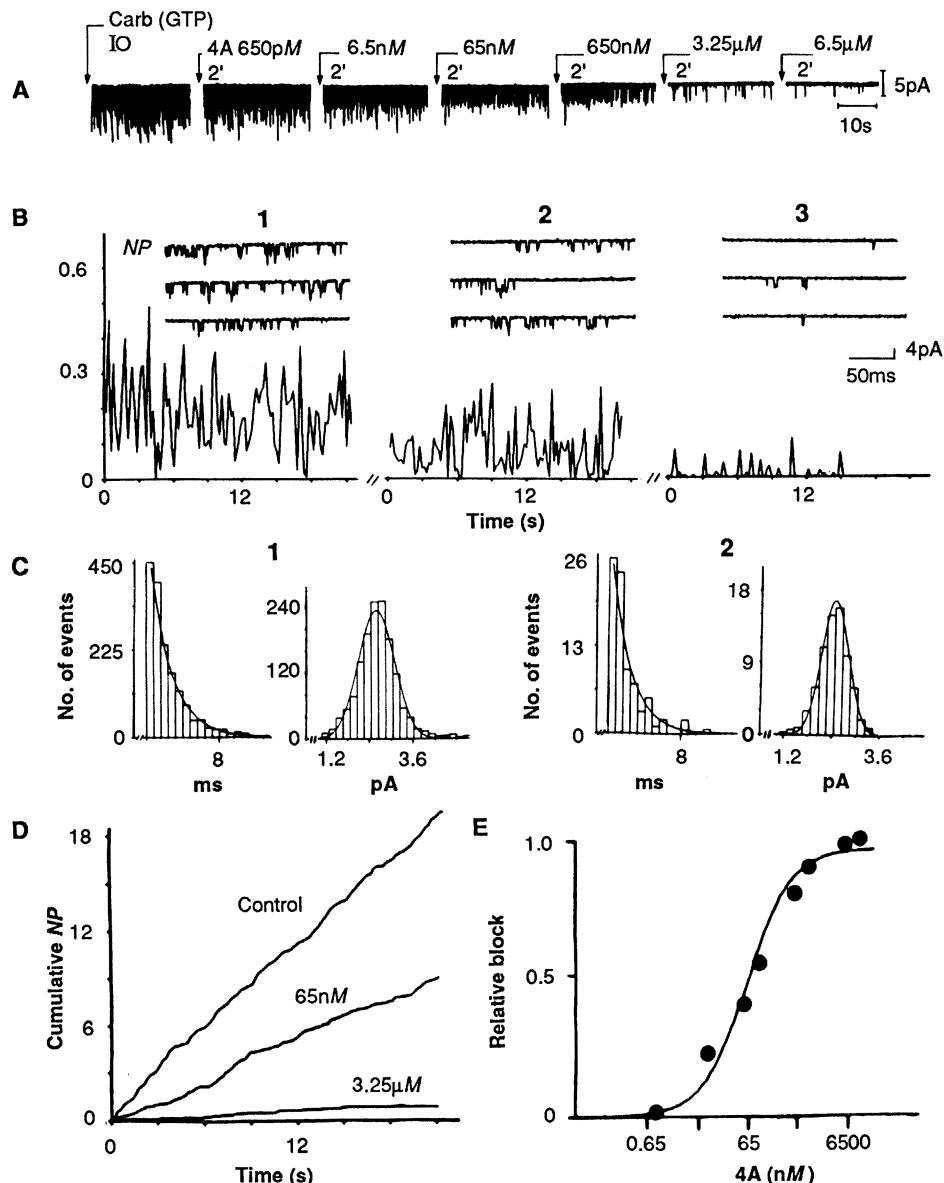


Fig. 3. mAb 4A block of the muscarinic acetylcholine activation of i_K is concentration dependent. (A) mAb 4A block is shown for the concentrations indicated. (B) 1, 2, and 3 are faster time resolutions of selected portions of the records in (A). i_K was stimulated by carbachol (Carb) (0.1 μM) in the patch pipette and GTP (100 μM) in the bath. Membrane potential was -100 mV. (B) also shows diaries of the proportion of open time per 0.5 ms (P) for the number of channels in the patch (N), that is, NP , on the ordinate as a function of time, in the absence (1) and in the presence of mAb 4A at 65 nM (2) and 3.25 μM (3). (C) Histograms of open times and amplitudes in control (1) and after 3.25 μM mAb 4A (2). The open-time time constants (τ) and SDs were 2.3 ± 0.06 ms (1) and 2.1 ± 0.02 ms (2). The mean and SDs of the amplitudes were 2.5 ± 0.02 pA (1) and 2.4 ± 0.03 pA (2). The resting membrane potential was -80 mV. (D) Cumulative NP is plotted against time before and after addition of mAb 4A at the two indicated concentrations. (E) The concentration-response curve of the effects of different concentrations of mAb 4A on i_K was normalized to the peak NP effect and fit to a one-to-one binding model with a K_d of 65 nM. Each point is the mean value from five to eight patches. SDs were within 10% of the means at each mAb 4A concentration. Data were obtained at membrane potentials of -80 or -100 mV.

K_{ACh}^+ channels; that is, formation of a tight complex between activated atrial α_k and the channel made the antigenic site inaccessible. Second, mAb 4A had no effect in nonactivating conditions, as illustrated by the absence of a persistent effect during incubation with membranes in which G_k was either inactive (carbachol present, GTP absent) or was activated at a rate too slow to produce significant stimulation of i_k (carbachol absent, GTP present). Steric hindrance from receptor-G protein coupling was ruled out in the agonist-GDP experiment (Fig. 2F) in which endogenous G protein was dissociated from the receptor (16), making the G protein accessible to antibody. We conclude that, in the patch clamp experiments, mAb 4A has a high affinity only for activated G protein.

Our results illuminate a plausible mechanism by which muscarinic agonists stimulate atrial K_{ACh}^+ channels. The physiological mediator of the G protein effect on K_{ACh}^+ could be (i) $\beta\gamma$ and not α (18), (ii) α and not $\beta\gamma$, and (iii) both α and $\beta\gamma$. The first was refuted by Codina *et al.* (8) and has now been refuted by its original proponents (19). Of the remaining two possibilities, our results favor α and not $\beta\gamma$.

On the basis of our data, mAb 4A blocks activated atrial G_k in one or two ways: either by binding to the α subunit that is still associated with the $\beta\gamma$ dimer or by binding to the α subunit that has dissociated from the $\beta\gamma$ dimer. If the antibody binds to the holo-G protein, the $\beta\gamma$ contribution to the activation of the K_{ACh}^+ channel must be minor because the holo-G protein and the resolved α subunit are equipotent at concentrations 100 to 1000 times lower than those used with resolved $\beta\gamma$ dimers (8–10, 14, 18). The same argument applies if the antibody restores the G protein to its unactivated GDP form. The conclusion that the $\beta\gamma$ subunit contribution is minor applies only to K_{ACh}^+ channels, because $\beta\gamma$ dimers are obligatory for rhodopsin-mediated stimulation of the guanosine triphosphatase activity of α_t and liver G_i (20), for the interaction of α_t with rhodopsin (21), and for the interaction of the α subunit of G_o with brain muscarinic receptors (22). However, if mAb 4A blocks G_k activation by binding to the dissociated activated α_k , our results indicate that $\beta\gamma$ dimers formed in situ in excess of α (see above) do not activate the K_{ACh}^+ channel under physiological conditions of agonist, GTP-dependent stimulation and that α_k is essential for channel stimulation.

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- Monoclonal antibodies to α_t were generated, purified, and characterized as in (12). $\alpha\beta\gamma$, α_t , and $\beta\gamma$ were purified to greater than 95% purity from bovine rod outer segments (23). α_t was purified as in (24). Soybean trypsin inhibitor was from Worthington. Aliquots of 1 μ l containing the indicated amounts of purified proteins or subunits were dotted onto nitrocellulose and allowed to dry for 10 min. After incubating nitrocellulose paper in 50 mM tris-HCl, pH 8.5, 150 mM NaCl (TBS) containing 3% ovalbumin (OTBS) to block nonspecific binding, it was transferred to the same solution containing 0.1 mg/ml purified mAb 4A or 4H and incubated overnight at room temperature. After rinsing sequentially with TBS, TBS containing 0.1% NP40, and OTBS, the nitrocellulose was transferred to OTBS containing 10⁶ cpm/ml Protein A (ICN) for 3 hours, rinsed again, dried, and exposed to film overnight at room temperature.
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- The G proteins used in this study were purified from human erythrocyte membranes to better than 95% purity by Coomassie blue staining following the fractionation schemes described previously [J. Codina *et al.*, *J. Biol. Chem.* **259**, 5871 (1984)] with the exception that DEAE-Toyopearl was substituted for DEAE-Sephadex in steps 7 and 8. GTP γ S-activated G_k (G_k^*) was prepared by incubation at approximately 40 μ g of G protein per milliliter (500 nM) in buffer containing 50 mM MgCl₂, 1 mM [³⁵S]GTP γ S (0.2 \times 10⁹ cpm/ml), 100 μ M GTP, 0.1% Lubrol-PX, 20 mM β -mercaptoethanol, 1 mM EDTA, 30% (v/v) ethylene glycol, and 10 mM tris-HCl, pH 8.0, for 30 to 60 min at 32°C, followed by extensive dialysis in the cold against the same buffer without guanine nucleotides until molar ratios of GTP γ S to G protein of less than 2.0 were obtained. GTP γ S-activated α_k (α_k^*) was prepared from 50 μ g of G_k by activating with GTP γ S, followed by chromatography over DEAE-Toyopearl and extensive dialysis to remove unbound GTP γ S. Activation was in 1.5 ml of solution containing 1 mM [³⁵S]GTP γ S (0.2 \times 10⁹ cpm), 100 μ M GTP, 1 mM EDTA, 100 mM MgCl₂, 30% ethyleneglycol, 20 mM β -mercaptoethanol, 4% Lubrol PX, and 10 mM tris-HCl, pH 8.0, for 30 min at 32°C followed by 13-fold dilution with 1 mM EDTA, 1 mM dithiothreitol (DTT), and 10 mM tris-HCl, pH 8.0. Chromatography was over DEAE-Toyopearl (200- μ l bed volume in a 1-ml disposable syringe) equilibrated with dilution buffer plus 7.5 mM MgCl₂ at room temperature. After application of the sample, the column was washed with 1-ml aliquots of equilibration buffer without and with 60 mM NaCl. Most of the [³⁵S]GTP γ S eluted under these conditions. α_k^* activity was then eluted with three 250- μ l aliquots of equilibration buffer containing 200 mM NaCl; the $\beta\gamma$ subunits were retained in the column. Dialysis was at 4°C against 7.5 mM MgCl₂, 1 mM EDTA, 40 mM KCl, 1 mM DTT, and 10 mM tris-HCl, pH 8.0. The resulting preparations had between 20 and 80 μ g of α_k^* protein per milliliter (0.52 μ M) and contained between 0.8 and 1.5 mol of GTP γ S per mole of α_k^* .
- Single atrial cells were dissociated from adult guinea pig hearts by collagenase. The solution in the patch pipette contained 140 mM KCl, 2 mM EGTA, 2 mM MgCl₂, and 10 mM Hepes. The pH was adjusted to 7.3 with tris base. The bath solution had the same composition. Single channel currents were measured with the gigaseal patch-clamp method. Patch pipettes had tip resistances of 5 to 10 megohms. The experimental chamber was placed on a microscope stage; it had a volume of 100 μ l. Antibodies and G proteins were added to the static bath and were washed out by gravity flow. Nucleotides were treated similarly but were also added by perfusion. All experiments were done at 20 to 22°C. Single channel currents were recorded with a patch-clamp amplifier (EPC7, List, Darmstadt, F.R.G.) the output of which was directed to a DA pulse-coded modulator (PCM-1, Medical System, Greenvale, NY) and stored on videocassette tape for subsequent analysis. Single channel currents were analyzed with a laboratory computer (PDP 11/73, Digital Equipment Corp.). Briefly, the records were low-pass filtered at 2.5 kHz (~3 dB) and digitized at 5 kHz. Transitions between closed and open states were determined with an automated, interactive threshold detection program that found the half maximum amplitude of single unit openings. The events were idealized, that is, transitions from closed to open and open to closed were assumed to occur as steps. Histograms of amplitude and open time distributions were constructed from idealized unitary events. Amplitude histograms were fit to Gaussian functions and open times were fit to exponential probability density functions. Fitting used a maximum likelihood estimator and parameters were optimized with a modified Marquardt-Levenberg routine. To obtain concentration-dependent effects of mAb 4A, we averaged single channel currents during the steady state responses (usually 2 to 3 min after addition) by measuring the proportion of open time P for the N channels in the patch, together called NP . NP was integrated every 10 or 20 ms and could be averaged over any arbitrary number of multiples of this period. The averaged NP value measured in the absence of antibody and plotted against the concentrations. The concentration-response curve was fit to single occupancy Langmuir adsorption isotherms.
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